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# Unsaturated lipids protect the integral membrane peptide gramicidin A from singlet oxygen

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ABSTRACT

**In contrast to expectations that unsaturated fatty acids contribute to oxidative stress by providing a source of lipid peroxides, we demonstrated the protective effect of double bonds in lipids on oxidative damage to membrane proteins. Photodynamic inactivation of gramicidin channels was decreased in unsaturated lipid compared to saturated lipid bilayers. By estimating photosensitizer (boronated chlorine $e_6$ amide) binding to the membrane with the current relaxation technique, the decrease in gramicidin photoinactivation was attributed to singlet oxygen scavenging by double bonds in lipids rather than to the reduction in photosensitizer binding. Gramicidin protection by unsaturated lipids was also observed upon induction of oxidative stress with tert-butyl hydroperoxide.**


## 1. Introduction

Oxidative stress is involved in the onset and progression of a great variety of pathologies, including cardiovascular and neurodegenerative diseases, moreover, numerous data support its crucial role in aging [1–3]. Direct oxidative damage to cellular components provokes a number of secondary processes, in particular, those providing protection and repair at the level of tissues, cells and organelles. Although oxidative modifications of lipids, proteins and DNA have been separately studied in detail, a much less understood issue is their interference. The relationship of different targets of oxidative damage can be explored by studying, e.g., the effect of the degree of unsaturation of lipid tails on the extent of oxidation and functional injury of membrane proteins. Actually, oxidizability of lipids may stimulate protein oxidation (due to peroxidative burst and indirect protein modification through attachment of lipid breakdown products (e.g., hydroxynonenal, malondialdehyde, acrolein) to Lys, Cys, and His residues in proteins) [4–7], or protect the protein from oxidation due to scavenging of reactive oxygen species by double bonds in lipids.

According to results of experimental dietary modification of in vivo membrane fatty acid unsaturation, lowering the double bond content of cellular membranes protected tissues against lipid peroxidation and lipoxidation-derived macromolecular damage [8]. Increasing the unsaturation of rat heart mitochondrial membranes augmented levels of markers of protein oxidative damage – malondialdehyde-lysine, protein carbonyls, and N(e)-(carboxymethyl)lysine adducts – in mitochondrial proteins [9]. However, no correlation between the degree of unsaturation of acyl chains and oxidizability of proteins was found in [10].

Relevant studies in model systems showed that the addition of liposomes containing cardiolipin or other unsaturated lipids substantially diminished cytochrome *C* oxidation elicited in water solution by tert-butyl hydroperoxide (*t*-BOOH) or photodynamic

*Abbreviations:* BACE, chlorin $e_6$ 13(1)-N-{2-[N-(1-carba-*closo*-dodecaboran-1-yl)methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester sodium; BLM, bilayer lipid membrane; gA, gramicidin A; *t*-BOOH, tert-butyl hydroperoxide; PDT, photodynamic therapy; DPhPC, 2-diphytanoyl-sn-glycero-3-phosphocholine; DPhPG, 1,2-diphytanoyl-sn-glycero-3-phosphatidylglycerol; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol; DLoPC, 1,2-dilinoleoyl-sn-glycero-3-phosphocholine

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treatment in the presence of methylene blue [11,12], thus revealing the protective role of double bonds in lipids. However, no influence of the cardiolipin content on the photodynamically induced cytochrome *C* oxidation was found in [13]. Besides, slight acceleration of the photodynamic inactivation but no protection was observed with Na, K-ATPase and Ca-ATPase incorporated in the membrane composed of unsaturated lipids as compared to that of saturated lipids [14].

To examine the protective effect of unsaturated lipids on oxidizability of membrane proteins, we have chosen the pentadecapeptide gramicidin A (gA), which is known to form ion channels in lipid membranes [15]. It was earlier demonstrated that membrane conductivity mediated by gA can be suppressed by reactive oxygen species generated in different ways: by X-ray-induced water radiolysis [16,17], by the addition of organic peroxide (*t*-BOOH) in combination with ferrous ions and ascorbate [18], or by photodynamic treatment [19–21]. The suppression of the gA ion-translocating ability in all these systems was associated with the damage to tryptophan residues (each gA peptide contains 4 tryptophanyls) [22,23]. Noteworthy, only in the case of the gA photodynamic inactivation, the damage is caused preferentially by singlet oxygen [21], the major cell-killing agent in PDT. Based on the phenomenon of gA photodynamic inactivation, we developed a technique for estimating the photosensitizing and antioxidant efficacy through measurement of the degree of photodynamic inactivation of gA channels in planar bilayer lipid membranes (BLM) [24–30]. Bearing in mind the reduction of radiolytic inactivation of gA in unsaturated lipids [31], here we studied the effect of the double bond content on the gA photodynamic inactivation sensitized by the boronated chlorin $e_6$ derivative BACE (chlorin $e_6$ 13(1)-N-{2-[N-(1-carba-closo-dodecaboran-1-yl)methyl]aminoethyl}amide-15(2), 17(3)-dimethyl ester) – a recently found, very effective agent for PDT [32,33], and compared the results with the corresponding influence of unsaturated lipids on gA channels in the case of the *t*-BOOH addition.

## 2. Methods

### 2.1. Chemicals

cThe sodium salt of BACE, synthesized as described earlier [33], was a gift from V.A.Ol'shevskaya (Nesmeyanov Institute of Organoelement Compounds, Russian Academy of Sciences). Chlorin $e_6$ was obtained from Porphyrin Products (Logan, UT). Lipids – 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC), 1,2-diphytanoyl-sn-glycero-3-phosphatidylglycerol (DPhPG), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphatidylglycerol (DOPG), 1,2-dilinoleoyl-sn-glycero-3-phosphocholine (DLoPC) – were purchased at Avanti Polar Lipids (Alabaster, AL). Gramicidin A was from Sigma.

### 2.2. Macroscopic electrical current across planar bilayer lipid membrane

BLM was formed from 2% solution of DPhPC, DOPC, DLoPC, DPhPC/DPhPG (70/30 w/w), or DOPC/DOPG (70/30 w/w) in decane on a 0.5-mm diameter hole in the partition separating the Teflon cell with a buffer solution into two compartments [34]. The aqueous solution contained 100 mM KCl, 10 mM Tris, pH 7.4. All experiments were performed at room temperature (23–25 °C).

The electrical current was recorded under voltage clamp conditions. Voltage was applied to silver-silver chloride electrodes connected to agar bridges or placed directly into aqueous solutions in the Teflon cell. The current was measured using a Keithley 428 amplifier (Keithley Instruments) in gA photoinactivation experiments or an OES-2 patch-clamp amplifier (OPUS, Russia) in current relaxation experiments, digitized using an NI-DAQmx digitizer (National Instruments, USA), and analyzed using a WinWCP Strathclyde Electrophysiology Software developed by J. Dempster (University of Strathclyde, GB).

### 2.3. Sensitized photoinactivation of gramicidin channels in BLM

Gramicidin A was added from stock solution in ethanol to the bathing solutions at both sides of BLM and routinely incubated for 15 min with constant stirring. The addition of gA at a concentration of about 1 nM induced a current across BLM of the order of 1 μA. In the gA photoinactivation experiments, BACE was added from the concentrated solution in dimethyl sulfoxide to the aqueous bathing solution at the *trans* side of BLM (the *cis*-side is the front side with respect to a source of illumination) and was thoroughly stirred for 20 min. BLM was illuminated by single flashes produced by a xenon lamp with flash energy of about 400 mJ/cm$^2$ and flash duration <2 ms or continuous light produced by a halogen lamp ("Novaflex", World Precision Instruments, Sarasota, FL). A glass filter cutting off light with wavelengths <500 nm was placed in front of a lamp. According to the previously published data [21], a single flash induces a decrease in the gA-mediated current ($I$). The time course of the decrease in the current is, at a first approximation, a monoexponential function of time: $I(t) = (I_0 - I_\infty) * \exp(-t/\tau) + I_\infty$, where $I_0$, $I_\infty$ and $\tau$ are the initial current prior to illumination, the steady-state level of the current established as a result of relaxation after the flash, and the characteristic time of photoinactivation, respectively. The relative amplitude of gA photoinactivation $\alpha$ is defined as follows: $\alpha = (I_0 - I_\infty)/I_0$. Here the value of $I_0$ was equal to 0.4 ± 0.25 μA in all photoinactivation experiments, because $\alpha$ was shown to be sensitive to the value of $I_0$ [21]. The voltage of 50 mV was applied to BLM.

### 2.4. Current relaxation after voltage jump

In voltage-jump current relaxation experiments [35–37], the voltage of 50 mV was applied to BLM or removed at the zero time, which resulted in current relaxation from $I(0)$ to $I(\infty)$.The capacitive response of BLM in the absence of BACE was recorded in each experiment (control measurements of the current at voltage values used in the experiment). Then BACE was added to both sides of BLM and recordings of the current in the presence of BACE were analyzed after subtracting the control traces.

## 3. Results

Fig. 1A illustrates time courses of the gA-mediated current (the current $I$ was normalized to the initial level $I_0$) through BLM formed from decane solutions of totally saturated (DPhPC, curves 1 and 3) or unsaturated (DOPC, curve 2) lipid. It is seen that illumination of BLM with a flash of visible light in the presence of 1 μM BACE led to a decrease in the gA-mediated current, which was significantly less pronounced in the case of the DOPC membrane (curve 2), as compared to the DPhPC membrane (curve 1). No decrease in the current was observed upon exposure of the BLM to the flash in the absence of the photosensitizer (curve 3). The time courses of the current decrease after the flash were well approximated by monoexponential curves (red curves) with a characteristic time $\tau$ of 0.49 s (curve 2) and 0.40 s (curve 1). According to our earlier studies [21], $\tau$ is determined by the rate constants of formation and dissociation of gA channels, generally considered to be transmembrane dimers [15,38]. Importantly, the relative amplitude of gA photoinactivation ($\alpha$) was about twofold

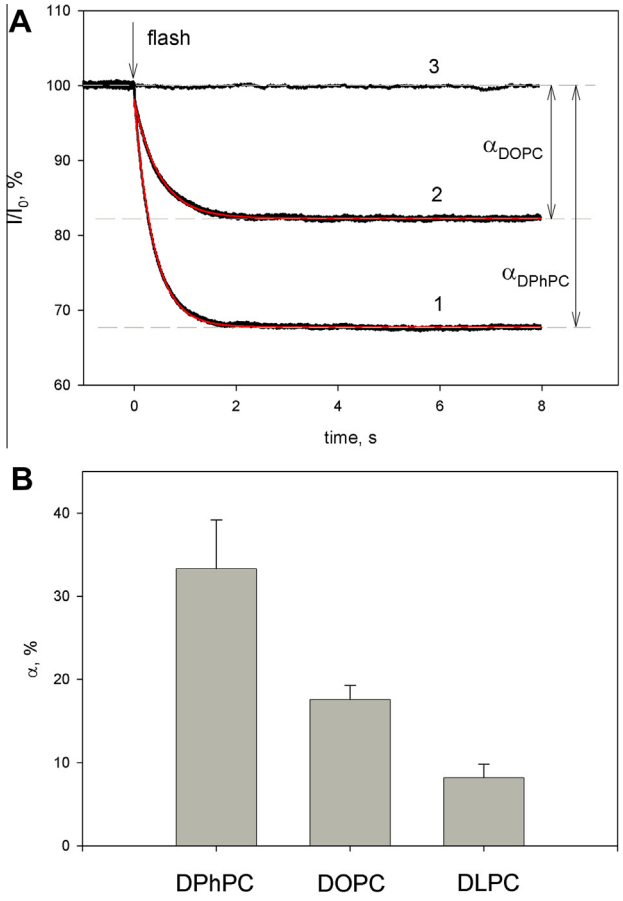


**Fig. 1.** (A) Time courses of the normalized electrical current ($I/I_0$) mediated by gA after illumination of BLM with a flash of visible light at $t = 0$ in the presence of 1 μM BACE (curves 1 and 2) or in the absence of the photosensitizer (curve 3). BLM was formed from DPhPC (curves 1 and 3) or from DOPC (curve 2). The initial current through BLM ($I_0$) was 0.39 ± 0.04 μA. Red curves show monoexponential approximation of experimental curves. (B) Values of the relative amplitude of gA photoinactivation α (Mean ± StdDev) resulting from illumination of BLM formed from DPhPC, DOPC or DLoPC with a flash of visible light in the presence of 1 μM BACE.

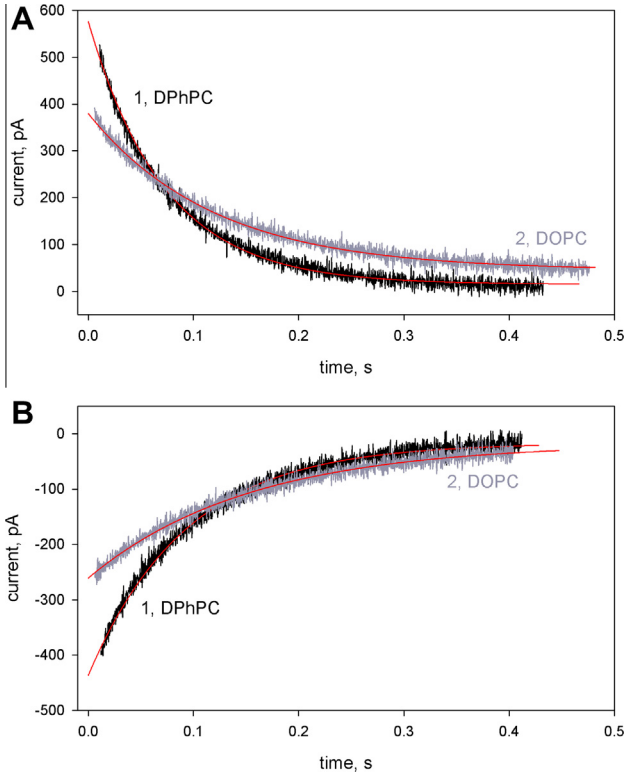


**Fig. 2.** Relaxation of the electrical current through BLM in the presence of 25 nM BACE elicited by a voltage jump to 50 mV (A) and back to zero (B) at $t = 0$. Red curves show monoexponential approximation of experimental curves. BLM was formed from DPhPC (black curves) or from DOPC (grey curves).

lower for the DOPC membrane with respect to the DPhPC membrane: 17.9% and 33.5%, respectively, thus demonstrating the protection of gA from the oxidative damage by double bonds in lipids. Fig. 1B displays the results of measuring α (Mean ± StdDev) for gA incorporated in membranes formed from DPhPC, DOPC and DLoPC. It is seen that increasing the number of double bonds in membrane lipid enhanced the protection of gA from the photodynamic damage. The reduction of α in the unsaturated versus saturated lipid bilayers was also found with unsubstituted chlorin $e_6$ (data not shown). In our earlier work [21], we mentioned the reduction of α in asolectin membranes (with rather high degree of unsaturation), as compared to that in DPhPC membranes, for gA photoinactivation sensitized by trisulfonated aluminum phthalocyanine. However, this result could be ascribed to a decreased binding of the anionic photosensitizer to the negatively charged surface of asolectin membranes. Some deceleration of the gA photoinactivation kinetics in the presence of double bonds could be associated with a decreased dipole potential of bilayers composed of unsaturated lipids, compared to that of saturated lipid bilayers [39]. This assumption is supported by our previous data on the effect of dipole potential on the gA photoinactivation kinetics [40].

Bearing in mind that photosensitizer binding to membranes is a prerequisite for the effective photodynamic modification of membrane-embedded targets [24], such as gA channels, we compared adsorption of BACE to DOPC and DPhPC bilayers by the voltage-jump current-relaxation technique [35,41]. This approach is applicable to measure binding to BLM of those charged compounds, which are capable of permeating through lipid bilayers due to delocalization of their charge. This capability of BACE shown in our previous studies [32,36] is obviously associated with the presence of a polyhedral carborane in its structure. Fig. 2 displays the current relaxation recordings following voltage jumps (switching on (A) and off (B)) of 50 mV across BLM formed from DPhPC (black curves) and DOPC (grey curves) in the presence of 25 nM BACE. The recordings were well described by monoexponential curves (red curves in Fig. 2), in line with the corresponding earlier data for such hydrophobic ions as tetraphenylborate and dipicrylamine [35,41–44]. With DOPC membranes, the current relaxation was a little slower (with the relaxation time constant being 120 ms upon application and 150 ms upon removal of the 50 mV voltage) than with DPhPC membranes (70 and 90 ms, respectively). However, the total number of ions moved through the bilayer during a single current transient, estimated from the area under the relaxation curve [35,36] in response to a voltage jump of 50 mV applied 20 min after the addition of BACE to the bathing solution, was similar for DOPC (37 ± 4 picoCoulombs) and DPhPC (38 ± 3 picoCoulombs) membranes (the mean value for 3 BLMs in each case). Therefore, the quantity of charged BACE molecules adsorbed on the bilayer boundary was the same for the unsaturated and the saturated lipid membranes, and thus a change in membrane binding of BACE could not be responsible for the decreased gA photoinactivation in DOPC membranes compared to DPhPC membranes. The surface concentration of the membrane-bound BACE ($N$) estimated from the equation $N = Q/(q * S_m)$ (where $Q$ – the total charge moved through the bilayer, $q$ – the charge of an electron, $S_m$ – the area of the membrane) amounted to $1.2 \cdot 10^{11}$ molecules/cm$^2$. The

difference between the relaxation time constants for BACE in DOPC and DPhPC membranes could be attributed (Fig. 2) to the difference in the membrane dipole potentials for bilayers formed from the unsaturated and saturated lipids [39]. Previously, we observed slowing down of the current relaxation kinetics for BACE in the presence of phloretin [36], the dipole modifier known to reduce the membrane dipole potential [45,46]. The sensitivity of transmembrane movement to the membrane dipole potential is a long established property of permeating ions, namely: the dipole potential facilitates permeation of lipophilic anions and mitigates permeation of hydrophobic cations through lipid bilayers [45–47].

The protective effect of unsaturated lipids on the sensitized gA photoinactivation was also examined with continuous illumination of BLM. Fig. 3 shows the time courses of a decrease in gA-mediated current across unsaturated (DOPC) and saturated (DPhPC) lipid membranes, which were recorded during illumination in the presence of 0.5 μM BACE. At high power density (0.77 W/cm$^2$) of illumination, the current across DOPC membranes rapidly decreased to zero after switching on the light (shown by an arrow) and then (after ~200 s of continuous illumination) began to grow in the course of illumination (Fig. 3, inset). The increase in the current, also observed with DOPC membranes in the absence of gA (data not shown), was apparently caused by lipid oxidation resulting in the formation of lipid pores [48,49]. With DPhPC membranes, no increase in the current was found under these conditions. The difference in the kinetics of the decrease in the gA-mediated current across unsaturated and saturated lipid bilayers was much more pronounced at low light intensity (0.18 W/cm$^2$). Initial rates of the current decrease amounted to 4.2 ± 1 and 14.9 ± 3.2%/s for DOPC and DPhPC membranes, respectively (mean values of 3 experiments with each lipid). In control experiments, illumination of DOPC bilayers in the absence of the photosensitizer (curve 3 in Fig. 3) did not provoke a decrease in the gA-mediated current. Suppression of the effect in the presence of the singlet oxygen quencher sodium azide (Fig. 3B) confirmed that the light-induced decrease in the current in the presence of BACE resulted from oxidative modification of a fraction of gA molecules by singlet oxygen, in line with our previous data obtained with sulfonated alumophthalocyanines [21].

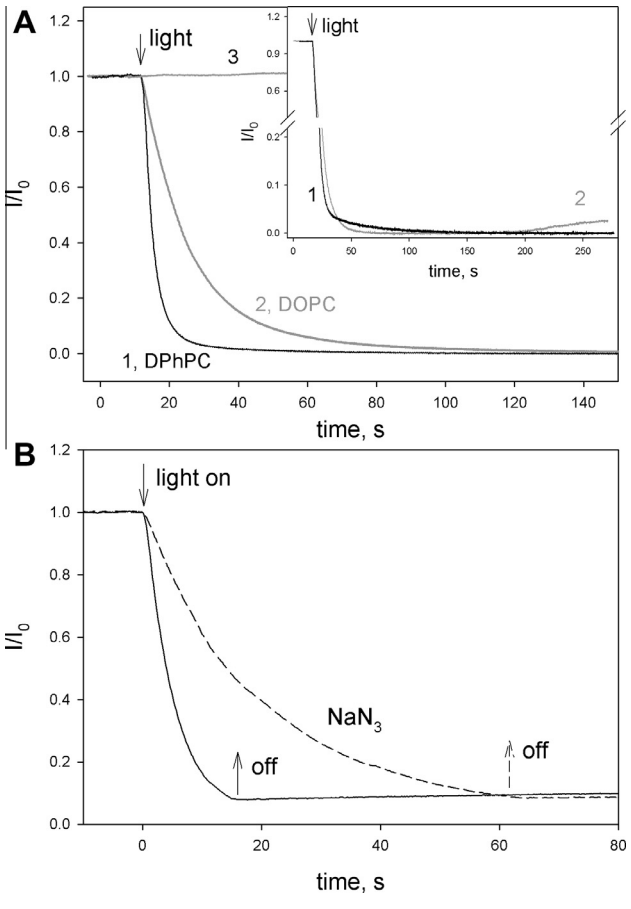


**Fig. 3.** (A) Time courses of the normalized electrical current ($I/I_0$) mediated by gA upon continuous illumination of BLM (0.18 W/cm$^2$, the beginning of illumination is shown by an arrow) in the presence of 0.5 μM BACE (curves 1 and 2) or in the absence of the photosensitizer (curve 3). Inset: time courses of $I/I_0$ at the power density of 0.77 W/cm$^2$ in the presence of 0.5 μM BACE. BLM was formed from DPhPC (black curves) or from DOPC (grey curves). The initial current through BLM ($I_0$) was 0.5 ± 0.17 μA. (B) Effect of sodium azide (20 mM) on the time course of the normalized electrical current ($I/I_0$) mediated by gA upon continuous illumination of BLM (0.18 W/cm$^2$) in the presence of 0.1 μM BACE. BLM was formed from DPhPC.

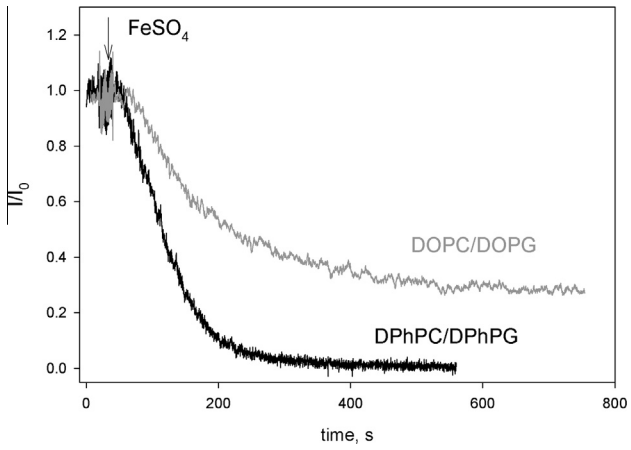


**Fig. 4.** Time courses of the normalized electrical current ($I/I_0$) mediated by gA after the addition of tert-butyl hydroperoxide (0.5 mM) in combination with ascorbate (0.5 mM) and $FeSO_4$ (20 μM). The moment of addition of $FeSO_4$ is shown by an arrow. BLM was formed from DPhPC/DPhPG (black curve) or DOPC/DOPG (grey curve).

As it was shown earlier [18], the addition of *t*-BOOH together with $Fe^{2+}$ and ascorbic acid to the water solution led to complete suppression of the gA-mediated ionic current across a DPhPC/DPhPG membrane, which developed in the minute timescale. With bilayers formed from DOPC/DOPG, the decrease in the current was substantially decelerated and did not reach zero (Fig. 4). Therefore, unsaturated lipids could also protect gA channels from the damage induced by organic hydroperoxide.

## 4. Discussion

Singlet oxygen generated as a result of energy transfer from excited photosensitizer to oxygen molecules plays a crucial role in photodynamic injury of cellular components [50]. According to [51], in animal cells, proteins containing oxidizable amino acid residues make the largest contribution to consumption of singlet oxygen, i.e. they represent the most abundant target for the singlet oxygen attack. In a series of studies [52–57], the impairment of structure and function of certain proteins was shown to be a key consequence of the photodynamic treatment of cells. However, singlet oxygen can also react with unsaturated acyl tails and cholesterol to give hydroperoxides, which in the presence of redox-active metal ions may turn over and propagate new chains [58]. It was shown that lipid peroxidation, caused by photodynamic treatment of model membranes containing unsaturated lipids, led to an increase in planar bilayer conductance [20,48], induction of leakage of fluorescent dyes from liposomes [30,49,59], and collapse of liposomal membrane potential [60].

The present study allowed us to gain insight into the relationship of oxidative modifications of membrane proteins and lipids in model membranes. The decrease in photosensitized inactivation of gA channels in unsaturated versus saturated lipid bilayers (Figs. 1 and 3) demonstrated that double bonds in lipids could protect the peptide from oxidative damage, apparently by scavenging singlet oxygen. Noteworthy, the increase in unselective membrane conductance obviously resulting from photodynamic lipid peroxidation required much higher doses of illumination than the blockage of gA channels (Fig. 3). The protection of gA exerted by unsaturated lipid upon induction of oxidative stress by organic hydroperoxide together with ferrous ions and ascorbate (Fig. 4) could be ascribed to radical scavenging capacity of double bonds in lipids [61].

The present study of the photodynamic inactivation of the channel-forming peptide gA revealed the protective role of unsaturated lipid surrounding integral membrane proteins, thereby supplementing the data on the similar protection observed with cytochrome *C*, the peripheral protein bound to the surface of negatively charged membranes [11,12].

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